

Xenobiotic Protection/Resistance Mechanisms in Organisms

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Glossary

Absorption In living organisms, the process by which environmental molecules move across biological membranes.

Adaptation The intergenerational genetically based selection of individuals for specific traits at the population level.

Avoidance The act of keeping away from or withdrawing from an undesirable environmental chemical.

Biotransformation The enzymatic conversion of a foreign or endogenous chemical to another form (metabolite) in living organisms.

Contaminant Any substance that enters a system where it is not normally found.

Defense A strategy or mechanism by which an organism protects itself or maintains its function in the face of a xenobiotic challenge.

Hydrophilicity Hydrophilicity refers to a physical property of a molecule that can transiently bond

with water (H₂O) (dissolve in it) through hydrogen bonding.

Hydrophobicity Hydrophobicity (Gr. *Water fearing*) is the physical property of a molecule that is repelled from a mass of water.

K_{ow} Octanol water partition coefficient, which generally describes the degree of hydrophobicity of a chemical. High Log K_{ow} values >4 are typical of hydrophobic chemicals.

Lipophilicity Lipophilicity (Gr. *fat-liking*) refers to the ability of a chemical compound to dissolve in fats, oils, lipids, and nonpolar solvents.

Phase I reactions Also called asynthetic or functionalization reactions and include oxidation, reduction, and hydrolysis reactions. In these reactions, a hydrophobic xenobiotic is usually converted to more water soluble metabolite.

Phase II reactions Also called synthetic or conjugation reactions in which a xenobiotic is covalently bound to an endogenous molecule forming a water soluble metabolite.

Phase III transport The products of Phase I or II biotransformation reactions and some parent xenobiotics are transported out of cells by various protein transport families collectively termed Phase III transport. In some cases, these transporters prevent the cellular entry of xenobiotics.

Pollutant A contaminant that adversely alters properties of the environment.

Resistance The capacity of a population of organisms to withstand the effects of a harmful environmental agent through genetic selection of less susceptible individuals.

Tolerance Reduced sensitivity of an individual organism, tissue, or cell to a foreign chemical and its toxic effects through acclimatory processes.

Toxicodynamics The process of the interaction of chemical substances with target sites (molecules, cells, tissues, or organs) leading to adverse effects.

Toxicokinetics Processes of the uptake of potentially toxic substances, their biotransformation, distribution, and elimination.

Xenobiotic Chemicals that are foreign to biological systems.

Definition of Subject

Organisms have always been exposed to chemicals that are foreign to them. Evolution has produced protective mechanisms against natural chemicals, mechanisms that are currently used against exposures to anthropogenically sourced chemical contaminants. Protection can occur at the individual level, often called tolerance, or at the population level, which is called resistance. The earliest studies on tolerance and resistance mechanisms come from the insect/pesticide literature; however recent environmental and health research is providing a wealth of information on these mechanisms in other organisms including humans. There are two major categories of protective mechanisms: (1) toxicokinetically derived mechanisms, which alter the way in which organisms absorb, biotransform, and excrete chemicals; and (2) toxicodynamically derived mechanisms, in which target sites are modified to reduce sensitivity. In both of these categories, protection can occur at the molecular and genetic level, through the cellular/tissue and organ levels, and up to and including whole organism responses including changes in behavior.

Introduction

Organisms have been exposed to foreign chemicals, or xenobiotics, through evolutionary history. The challenge of this chemical affront is to maintain appropriate concentrations of particular molecules to function, while excluding xenobiotics that will interact with cellular constituents. This is problematic, as organisms must remain open to a chemically dynamic environment for essential life processes. Chemical homeostasis employs cellular, physiological, and behavioral regulatory processes in response to the accumulation of xenobiotics that may cause departures from a state of dynamic equilibrium with the environment and reduce performance (e.g., reduced growth, reproduction) or

cause death. The early evolution of systems capable of reducing overall xenobiotic accumulation and their susceptibility to them, point to the evolutionary significance of xenobiotic protection mechanisms and their survival advantages.

Organisms have always been exposed to xenobiotics of natural origin. For example, host-plant resistance strategies often employ defensive secondary metabolites, known as allelochemicals (e.g., alkaloids such as nicotine, caffeine, morphine, colchicines, strychnine, and phenolics such as the cannabinoids), to influence the behavior, growth, or survival of herbivores. Herbivorous insects and mammals have evolved common protective defenses against the ingestion of allelochemicals. For example, Australian marsupials are constantly exposed to a diverse array of toxic xenobiotics that come from a wide variety of plant sources, such as *Eucalyptus* terpenes [1]. Marsupials display several significant xenobiotic processing mechanisms at the biochemical level for enhanced terpene detoxification, which is critically important for surviving on their unique *Eucalyptus* diet.

Organisms have adapted (to various degrees) to xenobiotic exposure of natural origin by evolving chemical defense/protective/resistance mechanisms. The relatively recent phenomenon of industrial pollution and the release of novel synthetic compounds into the environment at high concentrations and relatively short durations has not allowed for much evolutionary adaptation (although some) to synthetics. Most organisms rely on constituent chemical defense mechanisms for protection against this new chemical arsenal. The dramatic and catastrophic effects of pollution on many species points to the inadequacy of such defenses; however, in some cases the protection afforded by such mechanisms works equally well for anthropogenic chemicals. For example, the Great Lakes and Puget Sound, WA, have accumulated tons of PCBs, PCDDs, and PCDFs; yet despite chronic exposures to such compounds at concentrations that elicit toxicity in mammals and birds, populations of fish can flourish at very polluted locations. Although species diversity at these contaminated sites is reduced, signs of overt toxicity, with the exception of carcinogenesis, are rare. It is believed that

populations chronically exposed to high or even moderate levels of these contaminants have evolved or developed resistance mechanisms to these compounds [2].

There are two main possible means by which organisms can reduce their sensitivity to xenobiotics; those that occur at the level of the individual through acclimatory processes (often termed tolerance) and those that occur on populations of organisms in which the genetic constitution of a population is changed in response to the chemical, so that a greater number of individuals are able to resist it compared to the unexposed population (often termed resistance). Acclimation occurs at the level of the individual, where organisms become more tolerant as a consequence of a prior exposure through genetic or epigenetic mechanisms. Tolerance brought about through acclimation is not transmitted across multiple generations, therefore it is believed to wane if exposure is removed, for example, in remediated environments.

Adaptation is the intergenerational genetically based selection of more resistant individuals at the population level that results in individuals contributing relatively more offspring to subsequent generations. In order for adaptation to occur, resistant phenotypes must be present in sensitive populations before exposure, albeit at low frequencies. After remediation, adaptations may also disappear from populations because the selective pressure has been removed, but this occurs slower than for the acclimation process. Examples of heavy metal tolerance in plants are common and demonstrate dramatic changes in susceptibilities over a few generations. The evolution of industrial melanism in moths is an example of resistance that is widely cited as an example of contamination as a selective pressure for evolution.

Insect resistance to pesticides has been a well-studied area in this regard due to its scientific, economic, ecological, and health significance; pesticide resistance is a significant cause of failures in agricultural and pest management programs. Resistance to insecticides was first documented in 1914 by A. L. Melander for scale insects to an inorganic insecticide. Between 1914 and 1946, 11 additional cases of resistance to inorganic insecticides were recorded. Since

1945, it has been estimated that between 500 and 1,000 species of pests have developed a resistance to a pesticide. It was believed that the development of organic insecticides such as DDT would prevent insecticide resistance; however, by 1947 DDT resistance was exhibited by houseflies. With the introduction of every new insecticide class – cyclodienes, carbamates, formamidines, organophosphates, pyrethroids, even *Bacillus thuringiensis* – cases of resistance surfaced within 2–20 years [3].

Xenobiotic resistance is also significant in the area of human health. It is a major cause of failure of human therapies and has implications in antibacterial, anticancer, antipaludic, and antihuman immunodeficiency virus-1 (HIV-1) therapies. The mechanisms behind this resistance are conserved among bacteria, eukaryotic cells, parasites, and viruses [4].

Mechanisms of Resistance

Organisms use a variety of mechanisms to avoid the effects of toxic substances. These strategies range from the molecular to the behavioral level. Several different mechanisms have been proposed whereby resistance to chemical contaminants can develop in animal populations. The literature cites several classification schemes; however, here they are organized according to:

1. Mechanisms of decreased exposure through toxicokinetically derived resistance. Specific mechanisms in this category include reduced uptake or increased elimination of toxicants, thereby decreasing body burdens. Increased metabolic transformations of xenobiotics to less toxic or persistent forms can contribute to resistant phenotypes. Also, the sequestration of contaminants within cellular organelles or binding to proteins are means by which toxicants are unable to exert their toxic effects despite their accumulation.
2. Mechanisms of decreased sensitivity through toxicodynamically derived resistance. Mechanisms here include: increases in target receptor insensitivity (target site resistance) through changes in receptor structure, up- or down-regulation of target receptors, or decreases in the responsiveness of signal transduction pathways. Circumvention is the

bypassing of an inhibited pathway through an alternate route; limited opportunities for this mechanism exist, however it can occur.

Toxicokinetically Derived Resistance Mechanisms

Reductions in Xenobiotic Uptake

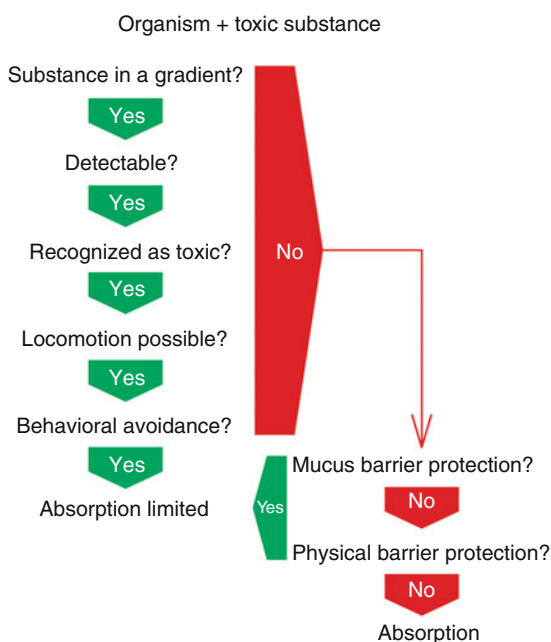
Limits to Exposure: Avoidable Exposures Ideally, organisms act to limit their exposures to harmful substances by sensing the substance and moving into a cleaner environment (Fig. 1). For this to occur, four conditions are necessary: (1) the substance must exist in the environment in a gradient, (2) the organism must be able to perceive the substance's presence, (3) the organism must identify the substance as harmful, and (4) the organism must be able to move

itself away from exposure. When met, these conditions provide a means of behavioral avoidance.

Environmental gradients – In aerial and aquatic environments of the past and present world, the first condition (a substance varying in concentration over space) is satisfied over small to large scales for innumerable toxic substances of natural and human origin. In the past, substances that organisms sought to avoid included hydrocarbons, toxins from plants and algae, and some arising from volcanic activity. Today, there is an ever increasing array of synthetics including pesticides, flame retardants, surfactants, and pharmaceuticals. Many of these synthetics are based on substances of natural origin, for example, pyrethroid insecticides are structurally similar to compounds of the chrysanthemum flower, and ethinylestradiol used in birth control pills is a more potent form of estrogen. Overall, there are few organisms that do not experience gradients of substances of both natural and synthetic origin.

Perception of environmental gradients – To meet the second condition of behavioral avoidance, organisms possess exteroceptive sensory mechanisms to gather environmental chemical and/or conditional information. Such mechanisms utilize intensity receptors, regardless of species. Intensity receptors can be relatively simple and exist at the molecular level in single-celled organisms such as in *Euglena* spp. [5], or more complex, such as those relying on multisensory neuron integration. The majority of our knowledge comes from the second category, for organisms with few sensory neurons (e.g., ~32 in *Caenorhabditis elegans*) [6] to millions (e.g., humans have ~six million olfactory sensory neurons alone) [7]. These sensory neurons rely on a diverse array of G-protein coupled receptors (GPCRs) that transduce molecular signals from the outside of the organism to the inside. The array of receptors in any given organism can be enormous – mice, for example, have 1,000–1,200 distinct olfactory receptors [8]. All intensity receptors have evolved to detect “natural” substances, and not novel compounds of human origin. Nevertheless, given the fact that many synthetic toxic compounds are based on naturally produced compounds, it is not unreasonable to expect “historic” sensory machinery to be able to function to some degree in the current environment.

Perceiving a substance as harmful – There are two routes by which a substance may be identified as



Xenobiotic Protection/Resistance Mechanisms in Organisms. Figure 1

There are many factors that determine whether an organism existing in a contaminated environment will end up absorbing any given contaminant. These factors include sensory and behavioral responses (e.g., the ability to detect a contaminant and move away from it), as well as the characteristics of the outermost structures (e.g., the permeability of keratin of skin and the chitin of arthropod cuticle)

harmful: through innately (genetically) recognizing it as so, or through acquiring (learning) its harmful nature. The former constitutes the most basic form of avoidance, that is, instinctive and not learned responses, which include reflexes and fixed action patterns (FAPs; a series of contiguous responses that continue until completion, e.g., sneezing). The latter involves memory and cognition, and so is restricted to organisms capable of these functions.

With innate avoidance responses, organisms respond aversively to unconditioned stimuli – stimuli that need not have been previously encountered. Reflexes, for example, do not even involve the brain (e.g., nociceptive withdrawal reflex to painful stimuli). Other innate responses may involve higher order neurological signal integration, such as in fishes that instinctively avoid sulfur dioxide and chlorine, which presumably helps maintain acidic and ionic balance [9]. Similarly, *Drosophila* instinctively avoids CO₂, apparently because CO₂ is associated with stressed conspecifics [10]. While these example substances can be increased in the environment as a result of human activities, their avoidance response is based on receptors and neural circuits. With synthetic, perceptible substances, there is no guarantee that an organism will identify them as harmful and so move down a concentration gradient. In fact, the opposite can occur – fishes are known to swim up concentration gradients of various pesticides, including bentazone, dalapon, and prochloraz [11]. Furthermore, innate responses may change over time, becoming reduced or enhanced in magnitude. Reduced responses can occur following successive exposures through neurological habituation (also referred to as “non-associative learning”). Decreased gill withdrawal response to painful stimuli is well documented in sea slugs (*Aplysia* spp.) [12]. Conversely, enhanced responses can occur with neurological sensitization. Drug seeking behaviors to amphetamine and cocaine are good examples [13]. What these innate response modifying mechanisms suggest is that over time, desirable avoidance responses may be lost or attenuated, and undesirable attraction responses may be enhanced.

In the absence of an innate response, organisms may limit future exposures to toxic substances by learning to avoid them. A mechanism by which this occurs is through associative learning, which occurs when an

organism learns to associate a conditioned stimulus (e.g., certain tastes or smells) with an unconditioned unpleasant or otherwise undesirable stimulus (e.g., pain). Through their pairing, the organism may even learn to respond aversively to the conditioned stimulus alone. This process is widely employed by organisms, using not only exteroceptive sensors, but also interoceptive sensors (i.e., those responding to internal stimuli). A well-known natural example of exteroceptive-based associative learning is the avoidance animals will show to skunks following an unfortunate interaction. Presumably this could occur for a toxic substance if an organism learned to pair it and its effects with a location or odor, although experimental examples are absent. A more tested form of learned avoidance is based on interoceptive responses. Ingestional aversive learning, for example, occurs when an organism ingests a specific food item, later experiences internally based pain or distress, and subsequently chooses to avoid that food in the future. Interoceptive avoidance learning is used by many organisms with naturally occurring toxins, from grasshoppers [14] to humans [15]. Furthermore, while associative learning was previously thought only to occur in organisms that possess neurological systems, recent evidence suggests the contrary: gene-regulatory networks appear to impart this ability in single-celled organisms [16]. Interoception is also the basis for the well-used conditioned taste aversion (CTA) assay. For CTA, a novel taste (conditioned stimulus) is typically paired with an injected toxin, and the rate at which the organism develops CTA is determined. This rate can be rapid in mice, just one pairing of the insecticide metabolite paraoxon with a novel taste was sufficient for development of CTA [17]. Clearly, should an animal survive an initial exposure to a toxic substance and associate a stimulus with that substance, future exposures have the potential to be avoided.

Innate and acquired avoidance responses may be adversely affected when an exposure is unavoidable and toxic substances are taken up (e.g., ingested) by the organism, especially if the substances are neurotoxic. Most of the studies of this type of impairment have focused on how natural avoidance responses are lost following contaminant exposure (e.g., the common toad (*Bufo bufo*) failed to behaviorally respond to chemical cues released by Turkish crayfish (*Astacus*

leptodactylus) following exposure to the herbicide amitrrole [18]. However, at least one study noted that contaminant exposure will modify future contaminant avoidance. Specifically, vendace (*Coregonus albula*) given weeklong exposures to bleached kraft mill effluent (BKME) and then subsequently given the choice to select clean versus contaminated water chose (preferred) BKME-contaminated water [19]. Predicting whether an organism will make the right choice when given the option to avoid exposure almost certainly requires empirical evidence.

Aversive locomotion – Those organisms with physical means to move in response to stimuli can do so by means of one of two sensory routes, and these are based on the complexity of their sensory mechanism(s). At the most simple extreme, the input from a single sensor can be used to guide locomotion. The resulting movement, referred to as klinotaxis, is evident in a behavioral phenotype characterized by “side-to-side” searching. This oscillatory motion is necessary for the organism to establish a gradient over the receptor, and therefore over the environment itself. Klinotaxis is generally understood to refer to positive motion, that is, up a gradient; however, the reverse is also possible. For more complex organisms, that is, those with paired intensity receptors, tropotaxis can occur. This motion is behaviorally evident in more linear movement, since the paired receptors can provide information of a concentration gradient across the location of the receptors, not just the environment. As for structures that enable movement, even single-celled spp. have the means, for example, *Euglena* spp. and its flagellum. A consideration is that locomotory ability is known to be highly sensitive to impairment by toxic substances, such as to the numerous anticholinesterase (anti-AChE) organophosphorus insecticides [20, 21].

Limits to Absorption: Unavoidable Exposures Arguably all organisms today receive exposure to synthetic substances. Toxic effects may ensue if any one or more of the four necessary avoidance conditions are not met and the substance passes into or through the external and internal coverings, that is, protective barriers, and into the tissues of the organism. Barriers include secretions, secretion clearance mechanisms, the epithelium, and other physical barriers to uptake.

Across phyla, organisms produce mucous to cover external and internal surfaces and cavities with an environmental interface. With vertebrates such as fish and mammals, goblet cells within mucous membranes are responsible for the production of mucus and are found in places such as the mouth, stomach, nose, and genitals. With invertebrates, including those in marine (e.g., *Polychaeta* worms) and terrestrial (e.g., slugs) [22, 23] environments, mucous is produced in mucous cells (mucocytes), which are in similarly sensitive areas of environmental exposure such as the malpighian tubules of arthropods [24]. Mucus fulfills multiple roles and contains constituents such as mineral salts, antibacterial enzymes, agglutinating glycoproteins (mucins), immunoglobulins, and other specialized proteins. These substances together form a mucous matrix, which serves as a potential “trap” for environmental substances, such as with tetracycline [25]. For vertebrates, proteins can be secreted into the mucous to achieve an additional function, namely, detoxification (i.e., biotransformation). Specifically, Phase II (conjugation) enzymes (e.g., glutathione S-transferases [GSTs]) can be found in mucus [26]. In sensory neurons, the presence of defensive biotransformation enzymes is critical not only for protection, but also to “inactivate” odorants and facilitate their removal [27]. Currently the presence of biotransformation enzymes in insect mucus remains to be explored, although biotransformation enzymes are known to be found in sensory organs (e.g., in the antennae of the moth *Mamestra brassicae*) [28]. Whether biotransformation occurs or not, for organisms with cilia, mucus (and any trapped substances) may be removed by mucociliary clearance, that is, propulsion of mucous away from cavities.

The outermost physical barrier of many organisms is covered with skin or epithelium, and fibrous protein or sugar-based coverings. Epithelium functions as a permeability barrier to toxic substances, limiting percutaneous absorption (cutaneous permeation), such as can occur with organophosphorus insecticides [29]. The resistance of skin is largely due to the stratum corneum, which contains dead cells rich in fibrous proteins known as keratin [30]. These dead cells are continuously sloughed off and replaced, sending any trapped substances with them. In many organisms, keratin is also found in more rigid and resistant

structures such as beaks, hooves, (reptilian) shells, and scales. In invertebrates and fungi, a barrier of polysaccharide termed chitin exists, and it has a similar strength to keratin. Chitin can be so formidable a barrier that numerous synthetic agents exist to inhibit its formation [31]. Nevertheless, outer barriers are not always uniform in strength; in fact there can be features that limit protective function. In mammals, for example, the pilosebaceous unit, which is composed of hair, its follicle, and the sebaceous gland, can provide an avenue of uptake [32]. Ultimately, the ability of outer physical barriers to prevent the uptake of foreign substances is a function of exposure concentration, duration, and the physicochemical properties of the substance.

Increases in Xenobiotic Elimination and Reductions in Accumulation

Biotransformation Biotransformation is defined as the conversion of a substance from one form to another by the actions of enzymes within an organism and is distinguished from physical-chemical processes that can also effect chemical conversions (e.g., photolysis). Biotransformation usually refers to the combined transformations, reflecting the reality that a particular chemical may undergo a number of chemical changes. A molecule or its biotransformational product, its metabolite, may be modified at a number of different positions with a variety of modifications, possibly resulting in several metabolites.

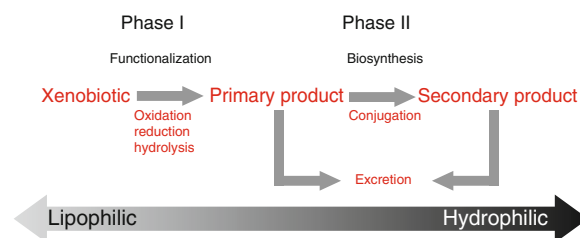
Xenobiotic metabolism is basically a process that introduces hydrophilic functionalities onto the foreign molecule to facilitate excretion. The availability of xenobiotics for biotransformation reactions and the rates of metabolism therefore have important implications on the tissue levels of toxicants, metabolite patterns, and xenobiotic half-life, all of which affect the bioaccumulation, persistence, and the severity and duration of a toxic response. For example, it has been shown that the four order of magnitude difference in the bioaccumulation of two structurally related compounds, DDT (2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane) and 2-bis(*p*-methylthiophenyl)-1,1,1-trichloroethane, with similar lipophilic characteristics, was due to the latter compound's ability to be biotransformed by the mosquitofish (*Gambusia affinis*) [33].

Hydrophobic (high log K_{ow} [octanol:water partition coefficients] value) and lipophilic chemicals

(e.g., PCBs, cyclosporine A) are not easily excreted, as they partition back into cells and tissues from excretory media (e.g., urine, bile). Water-soluble chemicals are not reabsorbed because lipid membranes of cells lining excretory routes act as barriers to their reuptake. This is why many lipophilic compounds tend to accumulate: they are easily absorbed and poorly excreted. For example, southern resident Killer Whales in the Pacific Northwest contain PCB concentrations as high as 146 mg/kg lipid weight, placing them among the most PCB-contaminated marine mammals in the world [34]. Therefore, the general scheme of biotransformation processes is the conversion of lipophilic compounds to more polar hydrophilic metabolites.

All organisms have the ability to metabolize foreign compounds that are taken up; however, their evolved abilities in this regard vary widely. The highest biotransformation ability is usually found in mammals and birds, followed by fish and reptiles, and invertebrates. Major sites of biotransformation include respiratory tissues, nasal mucosa, and the skin and gastrointestinal tract (all sites of potential entry of xenobiotics). The major site of biotransformation in organisms is the liver or functional equivalent. This organ has a high lipid content (to enhance lipophilic chemical accumulation), a high blood flow (increased delivery), and high titers of biotransformation enzymes.

Biotransformation generally occurs through a sequence of reactions termed phase I and phase II reactions (Fig. 2). In phase I reactions a nucleophilic functional group (e.g., $-OH$, $-SH$, $-NH_2$, $-COOH$) is



Xenobiotic Protection/Resistance Mechanisms in Organisms. Figure 2

The general biotransformation scheme for xenobiotics in organisms through phase I and phase II reactions. The general trend is to convert lipophilic compounds to more hydrophilic metabolites, which are more easily excreted

introduced or exposed in the parent molecule. In phase II reactions, the phase I metabolite or parent molecule already containing a functional group is conjugated to an endogenous molecule (e.g., sugar, amino acid, sulfate). Phase I metabolites are more polar than the parent molecule, increasing excretion potential, as well as rendering them suitable for phase II enzymatic reactions. Phase II reactions form exceptionally water-soluble products that undergo significant ionization at physiological pH. The endogenous conjugating moieties are also substrates for secretion proteins in transport epithelia. Phase I reaction products can be less toxic than the parent compound or may be bioactivated to more toxic metabolites (see below in oxidation reactions), a significant process in the toxicity of many environmental contaminants (e.g., dibrominated diphenyls) and drugs (e.g., acetaminophen).

Phase I Reactions (Asynthetic, Functionalization Reactions) Phase I reactions are the predominant biotransformation pathway for most xenobiotics and include microsomal monooxygenations, cytosolic and mitochondrial oxidations, co-oxidations in the prostaglandin synthetase reaction hydrolysis reactions, epoxide hydration, and reduction reactions. The common definition of Phase I reactions includes oxidation, reduction, and hydrolysis reactions, a grouping that lacks any mechanistic coherence. The modern systematic classification of enzymes (Enzyme Commission numbering system) groups together oxidations and reductions (catalyzed by oxidoreductases, EC class 1) but places hydrolysis reactions in a completely separate class (class 3). Not uncommonly, the term “phase I metabolism” continues to be used, mainly as an outdated synonym for P450-dependent oxidations. Regardless of nomenclature, the common theme to these reactions is to either unmask or introduce a polar functional group in or onto the parent xenobiotic.

Oxidation The cytochrome P-450 (CYP)-dependent mixed function oxidase (MFO) system of eukaryotes consisting of membrane lipids (e.g., mostly localized to the endoplasmic reticulum [ER], but also present in mitochondrial inner membranes), the enzyme cytochrome P450 (P450s), and an electron transfer chain (NADPH-cytochrome P450 reductase in the ER, and

redoxin reductase/redoxin in mitochondria) is crucial for oxidative, peroxidative, and reductive metabolism. P450s have two functions in general: (1) a protective role in the degradation or provision of polar handles for the solubilization of xenobiotics in preparation for excretion, and (2) a broad functional role in the biosynthesis of critical signaling molecules used for the control of development and homeostasis.

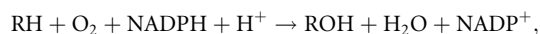
P450s constitute a superfamily of heme enzymes present in every living species on Earth from archaeobacteria to humans. Despite their occasionally minimal sequence similarity, all CYPs have a similar structural fold with a highly conserved core. Due to its high diversity, a systematic classification of individual CYP forms into families and subfamilies has emerged. The protein sequences within a given gene family are at least 40% identical (e.g., CYP2A6 and CYP2B6), and the sequences within a given subfamily are >55% identical (e.g., CYP2A6 and CYP2A7). Individual members of a family or subfamily are labeled again by Arabic numerals (e.g., CYP3A4, CYP3A7). There are 17 different families and 59 functional P450 genes currently known in humans. In this respect, hepatic drug oxidation is a major source of interindividual variation in pharmacokinetics and therapeutic response. The expression of individual P450 proteins in the liver is influenced by a number of factors, such as genetic make-up, disease, ageing, and environmental factors (e.g., smoking, alcohol, nutrition, pollutants) [35].

Early P450 research, suggested that only a few types of organic compounds served as P450 substrates, but it is now known that P450 substrates are an enormous and diverse group of compounds, including endobiotics and xenobiotics, and the list continues to grow. P450s do not conform to the typical textbook definition of an enzyme as a highly specific biological catalyst, and it has been predicted that the substrate base could be a million or more compounds [35]. Endobiotic substrates include such compounds as steroids, bile acids, fatty acids, prostaglandins, fat-soluble vitamins, amino acids, eicosanoids and retinoids, lipid hydroperoxides, and leukotrienes. Xenobiotic substrates include most of the therapeutic drugs and environmental contaminants, antioxidants, dyes, and plant products such as flavorants and odorants. The enzymes in the families 1–3 are mostly active in the metabolism of xenobiotics, whereas the other families have

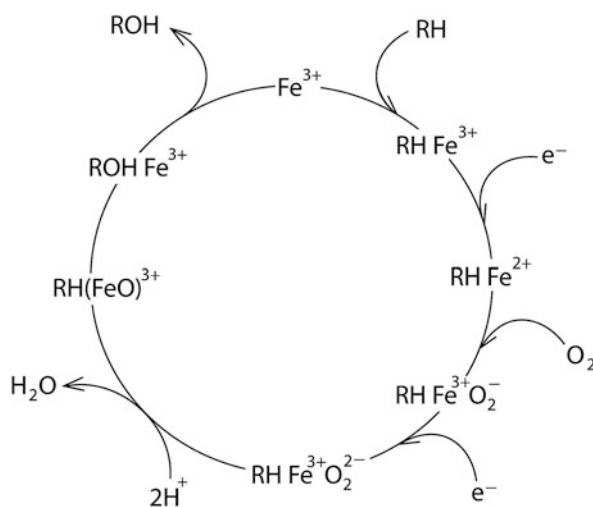
important endogenous functions. Interestingly, mutations that inactivate CYP enzymes with physiological functions often lead to serious diseases, whereas similar mutations in xenobiotic-metabolizing CYPs rarely do.

It should also be noted that P450 cytochromes occur in a variety of tissues other than liver. For example, P450 has been found in olfactory and respiratory nasal mucosa of animals, including fish.

Nature has found many ways to utilize atmospheric dioxygen to functionalize molecules for their degradation or synthesis through the use of a diverse set of cofactors. P450 is a heme (Fe at the active site) containing oxygenase, conscripted to metabolize atmospheric dioxygen in an oxygenase catalytic cycle (Fig. 3), resulting in the incorporation of one oxygen atom into a substrate. This hydroxylation reaction has the following overall stoichiometry:



where RH represents a xenobiotic or endogenous substrate and ROH is the product. The most important property of all known P450s is their ability to bind and activate two atoms of oxygen. A second characteristic property of P450s is that their active site is a heme macrocycle with the central atom, the heme iron, being bound to the protein structure through the anionic, thiolate sulfur of a cysteine residue. The



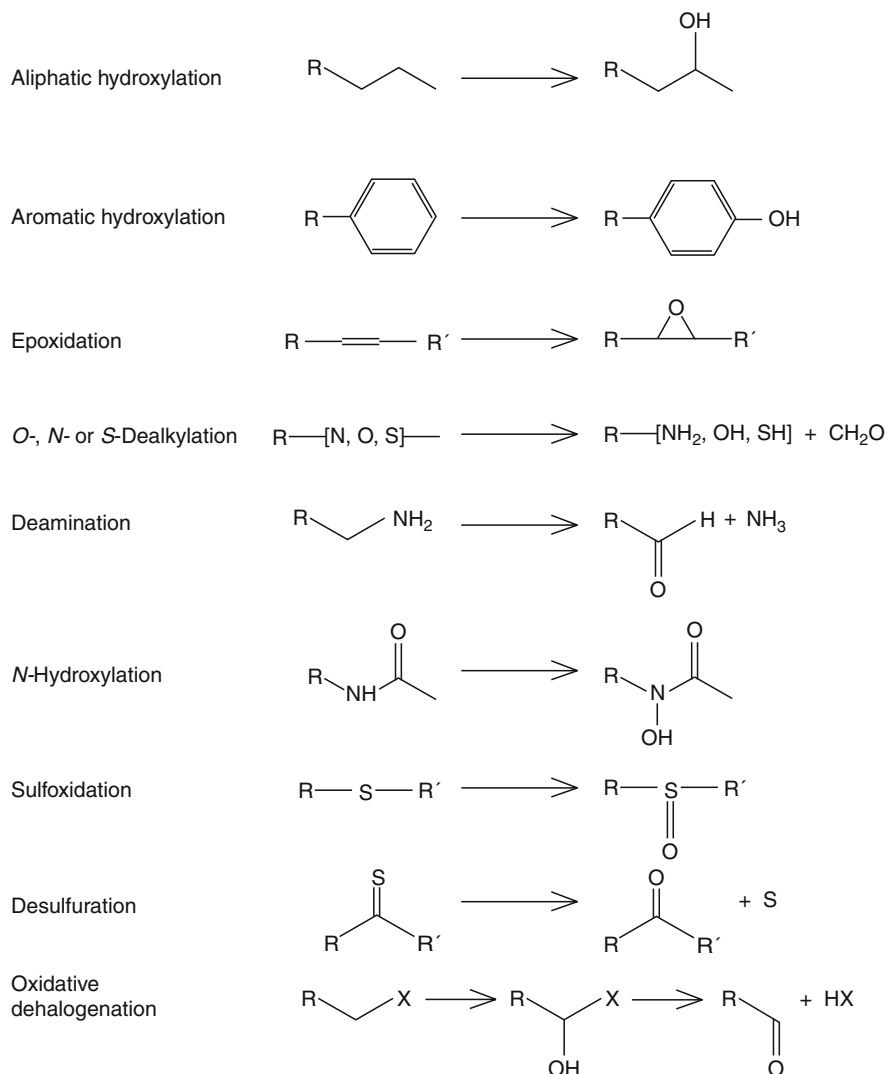
Xenobiotic Protection/Resistance Mechanisms in Organisms. Figure 3

The cytochrome P450 catalytic cycle

mode by which the heme is bound to apoprotein enables the ability of all P450s to activate the Fe–O–O moiety. The third common property of all P450 enzymes is their similar overall structure and shape, in which most of the secondary-structure elements, as well as the overall folding pattern, are conserved. NADPH-cytochrome P450 reductase uses NADPH as a cofactor that, after binding of the substrate to oxidized CYP P450, transfers electrons to CYP P450, thereby reducing it, allowing for several steps whereby one atom of oxygen from O₂ is transferred onto the xenobiotic and the other oxygen is used to form water. The oxygenation and other alterations of the very diverse number of substrates by P450s may seem indiscriminate, but in many instances the modification is positionally and even stereochemically specific. Some examples of reactions catalyzed by the microsomal cytochrome P450 system are given in Fig. 4.

Biotransformation generally results in reduced toxicity through the genesis of less toxic metabolites, although bioactivation to more toxic products (bioactivation) is a significant process in the toxicity of many compounds. Reactive intermediates can be formed directly through either phase I (oxidative or reductive reactions) or phase II reactions (less common), or by a rearrangement of a metabolite to an unstable form. Reactive intermediates are usually electrophilic and can react with nucleophilic sites on molecules such as the sulfhydryl group of glutathione and cysteine, or the guanine base in DNA. For example, the addition of oxygen to PAH C=C can produce an epoxide that can nonenzymatically react with macromolecular nucleophiles, resulting in covalent bond formation. Benzo[*a*]pyrene is a procarcinogen, and via a series of oxidation and hydrolysis reactions, a highly reactive metabolite (+)-benzo[*a*]pyrene-7,8-diol-9,10-epoxide is formed, which can react with cellular DNA and initiate the carcinogenic process [36].

Other important Phase I oxidative enzymes include flavin-containing monooxygenase, which catalyzes the oxygenation of nitrogen and sulfur compounds, and the epoxide hydrolases, which hydrolyze various epoxides to diols. The cytochrome P-450 and flavin-containing monooxygenase enzymes are localized in the microsomes, while epoxide hydrolases occur in both the microsomal and cytosolic fractions of the cell.



Xenobiotic Protection/Resistance Mechanisms in Organisms. Figure 4
 Typical oxidation reactions catalyzed by cytochrome P450 monooxygenases

Reduction Reduction reactions are also catalyzed by P450, owing to the transfer of reducing equivalents of P450 reactions to the xenobiotic substrate, rather than molecular oxygen. Reductions are most likely to occur with xenobiotics in which oxygen tension is low. Reductions can occur across nitrogen–nitrogen double bonds (*azo reduction*) or on nitro groups (NO_2). Frequently, the resulting amino compounds are oxidized forming toxic metabolites. Some chemicals can be reduced to free radicals, which are reactive with biological tissues.

Hydrolysis Hydrolysis reactions are typically not carried out by the P450 system. Grouping hydrolysis together with oxidation (as in phase I reaction) means separating hydrolysis from glutathione conjugation (phase II reaction), however, both hydrolysis and glutathione conjugation are reactions of an abundant cellular nucleophile (water or GSH) with a xenobiotic electrophile. In most cases, hydrolysis and glutathione conjugation are competing pathways for the disposition of xenobiotics (e.g., PAH dihydrodiol epoxides,

alkylating agents, *O*-acetoxy esters of aromatic amines, etc.) but they are certainly not sequential. The most significant hydrolyzing enzymes are carboxylesterases, arylesterases, cholinesterases, and serine endopeptidases. The hydrolysis of xenobiotic esters and amides generate carboxylic acids, alcohols, and amines. Such metabolites are all susceptible to phase II conjugation and excretion.

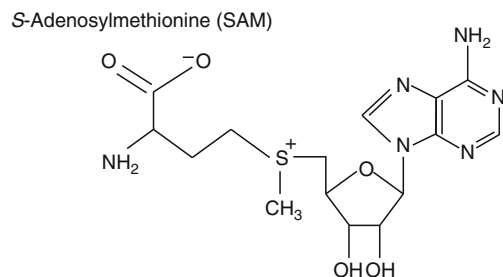
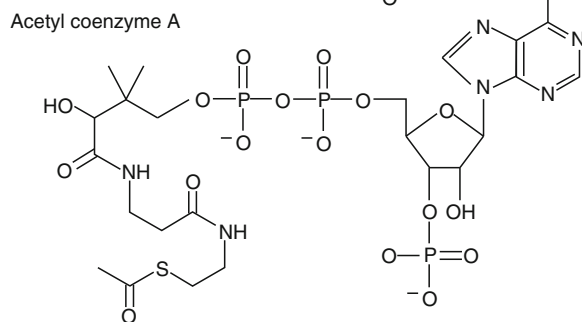
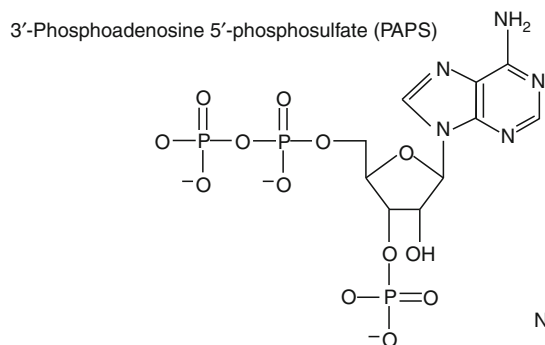
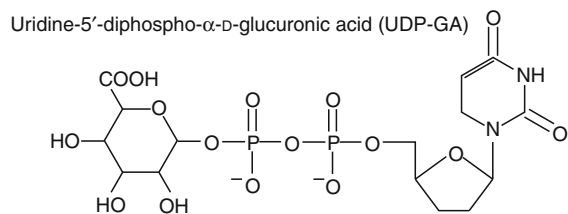
Phase II Reactions (Biosynthetic, Conjugation Reactions) Phase II reactions involve the biosynthetic union or coupling (conjugation) of xenobiotics or functionalized intermediates from phase I reactions with endogenous molecules generating hydrophilic conjugated metabolites that are devoid of toxicological activity and easily eliminated. Various endogenous molecules that are ionizable at physiological pH such as carbohydrates, proteins or amino acids, sulfur or phosphate components, or intermediates in lipid metabolism, are used in these reactions. Conjugated metabolites are substrates for specific transport proteins in epithelial tissues and are secreted with great efficiency. Conjugation reactions have a dual purpose; they have a role in intermediary metabolism of endogenous compounds as well as in the biotransformation of xenobiotics.

These reactions can be categorized by the mechanism of interaction between the conjugating agent and the xenobiotic. In type I reactions (e.g., glycosides, sulfates, methylated, and acetylated conjugates), ATP is used to form an activated “high-energy” intermediate. This activated coenzyme is conjugated to the substrate at the site of one of the functional groups. In type II conjugation reactions, the substrate itself is activated and then combines with an unactivated coenzyme (e.g., an amino acid), forming the phase II metabolite. In type III reactions, the xenobiotic or its metabolite already possesses the intrinsic reactivity to enzymatically react with a conjugating agent without the preformation of a high-energy intermediate (e.g., formation of glutathione conjugates). The enzymes that catalyze the reaction between the substrate and activated coenzyme are collectively called transferases. These reactions are usually considered as detoxication reactions, but in certain cases, bioactivation occurs (e.g., *N*-glucuronides of *N*-hydroxyarylamines are

transported to the bladder, where β -glucuronidase hydrolysis in the acidic urine produces hydroxylarylamines, which spontaneously form electrophilic arylnitrenium ions and cause bladder cancer [37]). Major phase II reactions include glucuronidation, sulfation, acetylation, and conjugation with glutathione or amino acids.

Glucuronidation Glucuronidation represents the major route of sugar conjugation, and qualitatively and quantitatively, glucuronide formation is the most important form of conjugation of both xenobiotics and endogenous compounds in most organisms. Four general categories of *O*-, *N*-, *S*-, and *C*-glycosides are formed and increase the compounds’ polarity and ultimately their excretion in urine or bile. In glucuronidation, the high-energy nucleotide uridine diphosphate (UDP)-glucuronic acid (UDPGA; Fig. 5) interacts with the functional group on the acceptor molecule (the substrate or aglycone) forming a conjugate with *D*-glucuronic acid. The transfer of glucuronic acid from UDPGA to the aglycone is catalyzed by a family of enzymes called UDP-glucuronosyltransferases (UGTs). There are multiple forms of UGTs that have different substrate specificities in the same species and in different species. For example, the domestic cat, *Felis catus*, can form glucuronide conjugates of thyroxine and bilirubin, but is unable to form other glucuronide conjugates. UGTs are localized to the ER of all tissues, but the majority of activity is found in the liver. Location in the ER is a strategy whereby it has direct access to products (including reactive intermediates) generated by microsomal P450, an excellent example of the integration of the sequestration of lipophilic xenobiotics in the lipids of the ER, the addition or unmasking of a functional group, and the conjugation of this functional group with the highly polar *D*-glucuronic acid.

Sulfation As with glucuronidation, these conjugation reactions are found in most organisms and are important to both endogenous (e.g., thyroid and steroid hormones, catecholamine neurotransmitters, the tyrosinyl group of peptides and proteins, and bile acids) and xenobiotic compounds, particularly those chemicals with hydroxyl groups, but also contributes to the biotransformation of 1°, 2°, and 3° alcohols,



Xenobiotic Protection/Resistance Mechanisms in Organisms. Figure 5

Phase II high energy cofactors

amines, and to a lesser extent, thiols. The resulting compounds are generally less toxicologically active and more polar, thus more readily excreted in the urine, with little in bile (this however, is species dependent). However, a number of compounds are converted to highly labile sulfate conjugates that form reactive intermediates that have been implicated in

carcinogenesis and other toxicities. Sulfate conjugation is a multistep process, comprising activation of inorganic sulfate, first, by converting it via ATP to adenosine-5'-phosphosulfate (APS), and further to the activated form, 3'-phosphoadenosine-5'-phosphosulfate (PAPS; Fig. 5) by ATP sulfurylase and APS kinase. PAPS is synthesized in all cells, but the highest concentrations are found in the liver or functional homologue in organisms without livers. Interestingly, the cat has very high levels of this conjugating enzyme system, perhaps compensating for the lack of glucuronidation as a primary conjugation mechanism. Sulfotransferases catalyze the reaction, whereby the SO_3^- group of PAPS is readily transferred in a reaction involving nucleophilic attack of the phenolic oxygen or the amine nitrogen on the sulfur atom with the subsequent displacement of adenosine-3',5'-diphosphate. Interestingly, sulfation is a high affinity, low capacity conjugation reaction, which is opposite to glucuronidation. At low phenol concentrations, sulfation predominates; at high concentrations, glucuronidation is the most important pathway. The major classes of sulfotransferases that metabolize xenobiotics are aryl sulfotransferases, alcohol sulfotransferases, tyrosine-ester sulfotransferases, and amine N-sulfotransferases.

Methylation Methylation involves the transfer of methyl groups from one of two methyl donor substrates, S-adenosylmethionine (SAM (Fig. 5)) or N^5 -methyltetrahydrofolic acid. Methylation is a common biochemical reaction of endogenous compounds (e.g., proteins, nucleic acids, phospholipids), but is not usually of quantitative significance in the biotransformation of xenobiotics. Methylation differs from most other conjugation reactions in that it masks functional groups, which may reduce water solubility and/or impair its ability to be excreted or participate in other conjugation reactions. N-methylation is an important reaction in metabolizing 1°, 2°, and 3° amines. Methyl groups from SAM are also transferred to the thiol-containing xenobiotics (e.g., thiopyrimidine drugs).

Acylation There are two types of acylation reactions based on the activated component. Acylation reactions involve foreign carboxylic acids and amines to form amide conjugates. In the first type, an activated

conjugating intermediate acetyl CoA (Fig. 5) reacts with a xenobiotic through acetyl CoA: amine N-acetyl transferases (mostly cytosolic enzymes) in a reaction called acetylation. Acetylation reactions require a specific cofactor, acetyl-CoA, which is obtained mainly from the glycolysis pathway via direct interaction of acetate and coenzyme A, or from the catabolism of fatty acids or amino acids. The primary site of acetylation is the liver, although extrahepatic sites have been identified as well (e.g., spleen, lung, and gut). This reaction has been studied extensively in humans because of its important consequences in drug therapy and in the carcinogenicity of certain xenobiotics.

In the second type of acylation reaction, amino acids are conjugated to carboxylic acid containing xenobiotics following activation of the foreign compound to form an acyl CoA derivative catalyzed by the enzyme acid:CoA ligase (a mostly cytosolic enzyme). The acyl CoA subsequently reacts with an amino acid (most frequently glycine, glutamine, alanine and histidine, and ornithine in birds, and taurine in fish) to form an acylated amino acid conjugate, catalyzed by N-acyltransferase. The reaction is categorized as a high affinity, low-medium capacity system, and given that it has only a relatively small substrate base such as aromatic, heteroaromatic and arylacetic acids, it is not considered as effective as the main conjugation reactions (glucuronidation, sulfate, GSH-conjugation).

Glutathione Conjugation and Mercapturic Acid Formation Glutathione (GSH) is a tripeptide that contains cysteine, glutamic acid, and glycine. In its reduced form, it acts as an antioxidant due to its nucleophilic nature at the sulfur group, and as such, it is essential in protecting cells from reactive oxygen species (ROS). GSH is found almost exclusively in its reduced form since the enzyme that reverts it from its oxidized form, GSH reductase, is constitutively active, and is present in high concentrations in all tissues, particularly the liver.

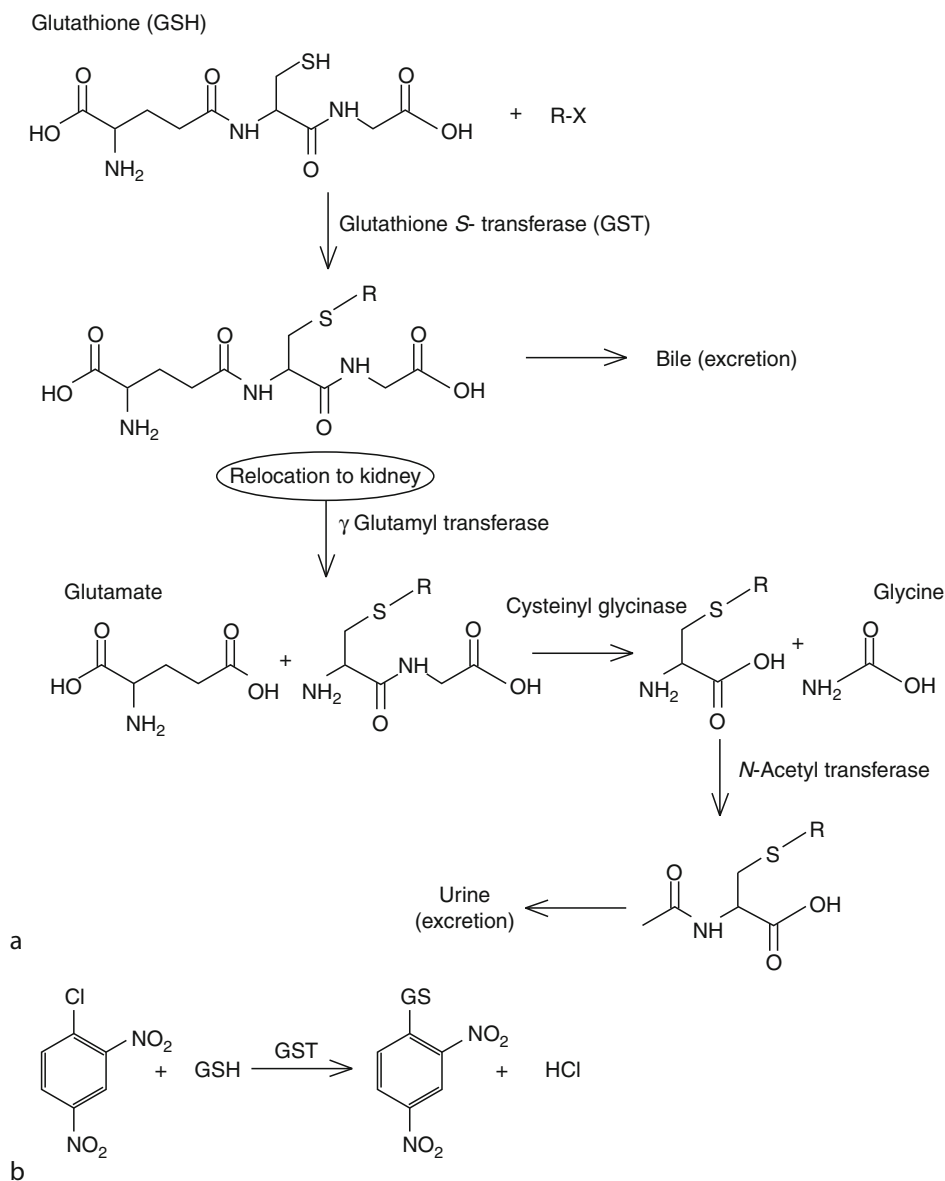
The glutathione S-transferases (GSTs) are a family of mainly cytosolic enzymes (microsomal and mitochondrial forms do exist) that play an important role in xenobiotic resistance by several distinct mechanisms: increased detoxication, sequestration of the xenobiotic, and decreased xenobiotic activation. Arguably the most important function is to catalyze the initial reaction in

the formation of N-acetylcysteine (mercapturic acid) derivatives of a diverse group of xenobiotics with the following characteristics in common: some degree of hydrophobicity, presence of an electrophilic carbon atom, and some tendency to react with glutathione nonenzymatically (e.g., electrophilic substrates including herbicides, insecticides, pharmaceuticals and anesthetics, anticancer agents, carcinogens, antibiotics, etc.) GSTs are found ubiquitously in all tissues, with the highest levels in the testes, liver, intestine, kidney, and adrenal glands of humans. They have also been found in every organism examined.

The initial step in mercapturic acid formation (Fig. 6, with example) involves the formation of a glutathione conjugate. GSH conjugation involves the formation of a thioether link between the GSH and electrophilic compounds. The reaction can be considered as the result of nucleophilic attack by GSH on electrophilic carbon atoms; thus conjugation with glutathione usually results in detoxication of electrophilic compounds by preventing their reaction with nucleophilic centers in other biomolecules. The enzyme γ -glutamyltranspeptidase, a membrane-associated enzyme found in high concentrations in cells that provide absorptive or excretory functions, removes glutamic acid from the GSH-conjugate, forming a cysteinyl glycine conjugate. Glycine is then removed from this metabolite by the enzyme cysteinyl glycine dipeptidase or aminopeptidase M, to form the cysteine S-conjugate (pre-mercapturic acid), which is acylated by N-acetyltransferases, resulting in the formation of the N-acetyl derivative, mercapturic acid.

Modifiers of Biotransformation The dramatic significance of biotransformation and the mechanisms to achieve the protection and resistance of organisms from the effects of xenobiotic exposure have been discussed. There are a number of endogenous (physiological) and environmental factors that affect the abilities of different organisms to use biotransformation in a protective role, factors which can modify their susceptibility to toxicants. Some of the more important intrinsic factors include the following:

- Species, strain, and other genetic variations (particularly interesting with individual sensitivities to either drugs or environmental contaminants from a health perspective)



Xenobiotic Protection/Resistance Mechanisms in Organisms. Figure 6

(a) Glutathione conjugation and mercapturic acid formation. **(b)** An example of glutathione conjugation

- Development (under complex ontogenic control)
- Sex (males typically > females)
- Age (low in fetus and infants, increasing in age and declines with old age)
- Hormones (complex interrelationship: antagonistic, additive, and synergistic relationships)
- Pregnancy (decrease in maternal enzymes)
- Disease (tissue and organ-specific as to magnitude, but generally decreases with hepatic disease, infections, cancer)
- Diurnal and seasonal cycles (direction with circadian rhythms depends mostly on the endogenous function of enzymes; season in wildlife usually reflects hormonal fluctuations)

- Nutritional effects (deficiencies generally decrease enzymes) [38]

Extrinsic factors that can modify biotransformation include: pre-exposure to xenobiotics, resulting in either inhibition of biotransformation enzyme systems or induction (increases) in enzyme concentrations following exposure. In ectothermic organisms, environmental temperature and the rate of temperature change can play a large role in altering xenobiotic metabolism.

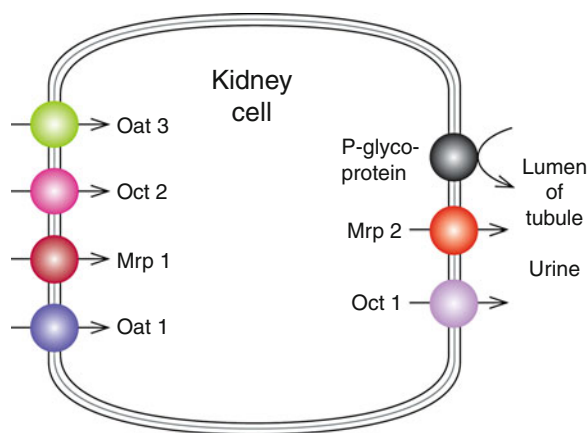
Prior exposure to some contaminants can increase the biotransformation capability of organisms by a process called *induction*. For example, P450 isoforms in the 1A (CYP1A) subfamily are inducible by planar PAH and HAH, including chlorinated biphenyls, dibenzo-*p*-dioxins, and dibenzofurans, as well as several drugs including the classic (well studied pharmacologically) inducers phenobarbital and 3-methylcolanthrene. The Ah receptor (AhR), a soluble protein with a high binding affinity for planar PAH and HAH, controls the induction of cytochrome CYP1A. The aryl hydrocarbon receptor nuclear translocator (ARNT) and the AhR together form a transcription factor complex through which altered gene expression and toxicity occurs [39]. Their dimerization is dependent upon high affinity binding of planar PAH and HAH to the AhR. The AhR:ARNT complex then binds specific genomic recognition sites (xenobiotic response elements, XRE) on DNA. This alters the transcription of target genes that include both phase I and phase II biotransforming enzymes. Other nuclear receptors are involved in the induction of biotransformation enzymes, and considerable current research effort is focused in this area. Like AhR, orphan nuclear receptors (e.g., constitutive androstane receptor [CAR], pregnane X receptor [PXR], and retinoid X receptor [RXR]) comprise a gene superfamily that encodes the transcription factors that sense endogenous (e.g., small lipophilic hormones) and exogenous (e.g., drugs, xenobiotics) compounds, and transfer this information into cellular processes by regulating the expression of their target genes [40]. The regulation and induction of phase II enzymes is less well understood, and recent research has revealed the existence of several *cis*-acting regulatory elements including the antioxidant response element (ARE/electrophile response element [EpRE]), xenobiotic responsive element (XRE/aromatic hydrocarbon responsive element

[AhRE]), activator protein-1 (AP-1), and nuclear factor-kappa B (NF-kB) binding sites. In the case of ARE, xenobiotics induce stress responses leading to potential sulfhydryl modification of Keap1-Nrf2 and other signaling pathways, which leads to the activation of the transcription factors such as Nrf2/Maf and increases ARE-mediated gene expression including phase II enzymes [41]. Maximum induction with chemicals is dependent on species, inducer (variation exists in inducer strength), route of administration, and dose; however, induction can occur quite rapidly (within hours in *in vitro* assays, days *in vivo*), and enzyme activities can remain elevated for weeks upon withdrawal of the inducer.

Unlike induction (a physiologically regulated and evolved process for protection), the inhibition of biotransformation enzymes can include a multitude of mechanisms including competition for active sites or cofactors of enzymes, decreased biosynthesis, increased degradation of enzymes or cofactors, allosteric changes in enzyme conformation, loss of functional tissue, etc.

Phase III Transport In many cases, these transporters act as a first line of defense, preventing toxic chemicals from entering the cell. However, if a compound is not recognized by these transporters and enters the cytoplasm, detoxifying enzymes in the cell may modify the chemical to a more hydrophilic form. In this case, related cellular transporters can again come into play, effluxing the modified products out of the cell. There are two main types of these: *ATP-binding cassette* (ABC) and *solute carrier* (SLC) proteins (Fig. 7).

ABC Proteins ABC transporters are evolutionarily ancient, and three ABC subfamilies of efflux (multidrug) transporters involved in the biochemical defense against toxicants have both health and environmental significance [42]. ABC drug efflux transporters have a wide phylogenetic distribution and are found in vertebrates as well as in deuterostome invertebrates, protostome invertebrates, fruitflies, protozoans, and yeast. Homologous proteins are also present in plants, though their roles in multidrug efflux have not been firmly established. ABC transporters include members of the P-glycoprotein (P-gp [ABCB]) family, the multidrug resistance protein



Xenobiotic Protection/Resistance Mechanisms in Organisms. Figure 7

Vectorial transport of xenobiotics and their metabolites is accomplished by efflux transporters as pictured in this kidney cell. *Oat* Organic anion transporter, *Oct* Organic cation transporter, *Mrp* multidrug resistance-associated protein

(MRP [ABCC]) family and the breast cancer resistance protein (BCRP or MXR [ABCG]) family. These ABC proteins are membrane-bound transporters coupling ATP hydrolysis to the translocation of substrates across biological membranes. These efflux pumps provide important protection to internal tissues (e.g., blood–brain barrier, liver, kidney, and placenta) and to sites of entry of environmental contaminants (e.g., gills, gastrointestinal tract).

P-Glycoprotein (ABCB Family) The multidrug resistance (MDR due to its remarkably nonspecific substrate base) protein, P-glycoprotein (P-gp for “permeability glycoprotein,” [ABCB1]), transports both endogenous and exogenous substrates and is found in a broad range of taxa [43]. It is a transmembrane energy-dependent pump that mediates the efflux of a large number of moderately hydrophobic compounds. P-gps confer MDR to tumor cell lines [44] and tumors of human patients [45], and have been described in many aquatic wildlife species [46]. It appears that, in addition to normal cell function, P-gp activity contributes to the relative hardiness of fish living in contaminated environments, providing multixenobiotic resistance (MXR).

Although the high resolution structures of ABC proteins and the structure–function relationships of mammalian P-gp have been characterized in recent years, how it works as an efflux pump or its role in normal physiology is still unknown. A role for P-gp certainly exists in altering the pharmacokinetics of natural endogenous compounds and xenobiotics, although the full range of substrates for P-gp has not yet been explored. The factors that regulate P-gp are also not yet fully understood, and understanding the regulation of these efflux pumps is essential in determining their role in normal physiology. Limited evidence suggests P-gp is up-regulated (induced) in response to a number of signals, including specific substrates, toxicants, heat stress, and nuclear factors [47].

P-glycoproteins are expressed in many of the same tissues as biotransformation enzymes (e.g., cytochromes P450); however, biotransformation is not a prerequisite for efflux. Compounds are transported as parent molecules, and among P-gp substrates, no consensus structure has been defined, although within each class of chemicals certain chemical features have been defined that seem to be essential for functional interaction (moderate hydrophobicity, small size, and positively charged domains). Their interaction with biotransformation systems, or other transporters (e.g., multidrug resistance protein [MRP]) are unclear. Although it is now clear that the induction of CYP enzymes and P-gp are not co-regulated, they may interact through CYP-mediated production of P-gp substrates or CYP-mediated- or glutathione (GSH) depletion-mediated increases in intracellular reactive oxygen species (iROS) [48]. As a first line of cellular defense against xenobiotic influx, P-gp may be overwhelmed by high concentrations, or may not recognize certain classes of compound, which enter cells and are acted on by biotransformation enzymes such as P450, followed by the conjugation of the metabolite. The conjugated metabolite can then be eliminated by another ABCC family of efflux transporters, which recognize the conjugated moiety (e.g., MRP).

Multidrug Resistance-Associated Protein (ABCC Family) Conjugates require export across cell membranes, and conjugated metabolites may be subject to further metabolism (e.g., the pathway of mercapturic acid formation) or transported without

further modification out of cells. The use of the term “Phase III metabolism” to describe membrane transport of drug conjugates [49] is also potentially misleading, since proteins such as MRP1 effect transport of some drugs in their parent form, without any prior biotransformation, and actually prevent cellular uptake. Some members of the multidrug resistance-associated protein (MRP, ABCC) family also contribute to multidrug efflux by acting like P-gp to efflux unmodified xenobiotics. However, members of this family also act on endogenous substrates that are normal products of metabolism, or act on xenobiotics that have been modified as a part of the above mentioned phase I and phase II biotransformation processes. The resulting conjugated, negatively charged molecules are recognized by various ABCC transporters and then exported from the cell [48].

Multixenobiotic Resistance Protein (ABCG Family) ABCG2, a member of the ABCG family commonly referred to as breast cancer resistance protein (BCRP), also causes significant resistance to a limited group of chemotherapeutic treatments (substrate specificity is less broad than for ABCB and ABCC [50]) as well as effluxing dietary toxicants [51]. A difference from the aforementioned transporter types, which are so-called full transporters (the gene product constitutes one structural unit), is that ABCG2 is a half transporter, in which two protein molecules are assembled to form a structural unit active as a homodimer. ABCG2 also plays a role in the translocation of essential compounds, such as riboflavin, into breast milk and may also transport certain toxicants into milk. In fact, in an environmental sense, transfer of milk to offspring of top predators such as whales, puts calves at a higher trophic level than the mothers, making young the highest risk group [52].

Multixenobiotic Resistance (MXR) MXR is a phenomenon that increases the potential for survival of aquatic organisms in polluted environments through enhanced expression of one or more MDR-like transport mechanisms (e.g., P-gp or MRP) that restrict xenobiotic uptake [53]. As mentioned earlier, these transporters act as first lines of defense against xenobiotics and are found in tissues where exchange with the environment can ensue. Such

transport systems are present at some basal level in animals maintained in unpolluted environments, and they appear to be up-regulated on exposure to pollutants.

Mixtures of xenobiotics can compromise this defense because competitive binding of different chemicals to the transporters can sabotage the activity of transporter binding sites or saturate them. In addition, some of the chemicals in the mixture might be direct inhibitors of the transporter activity. The resultant competition or inhibition, termed chemosensitization, can decrease transporter activity of chemicals in a mixture such that toxic substances normally excluded from the cell can now enter.

Solute Carrier Proteins Elimination in most organisms occurs via the kidneys and hepatobiliary route or functional equivalent. In kidney tubules and in hepatocytes, as well as other tissues, there exist a number of other xenobiotic (or metabolite) export pumps that mediate the vectorial transport of substrates from the basolateral extracellular fluid into the cells on the basolateral sides of cells and export them to excretory media on the luminal side of cells (e.g., facing canaliculi or lumen) (Fig. 7). Some of these transporters include the aforementioned ABC proteins. At the basolateral membrane are members of the SLC superfamily of proteins, the organic anion transporters (Oat1, Oat3), and organic cation transporters (Oct2). Oat1 exhibits a broad substrate specificity and is responsible for the cellular uptake of most amphiphilic organic anions. High levels of expression of organic cation/carnitine transporter (OCTN1) are seen in barrier and excretory tissues as well [54].

Regulation of Efflux Transporters Pregnane X receptor (PXR), constitutive androstane receptor (CAR), peroxisome proliferators activated receptors (PPAR), liver X receptor (LXR), and farnesoid X receptor (FXR) are all nuclear receptors that bind to specific DNA response elements that produce effects on phase III efflux transporters, although which specific transporters and the mechanisms are still unclear [41]. PXR appears to regulate several efflux transporters including P-gp, MRPs, and Oats. Common mechanisms exist between phase I and phase II xenobiotic metabolizing enzymes through AhR/ARNT, and binding to AhR-xenobiotic

response elements and subsequent induction of mRNA suggesting coordinate regulation. Moreover, several types of phase II enzyme inducers have been empirically shown to increase the excretion of phase II metabolites. Since phase II metabolites are transported out of cells by P-gp, MRPs, and OatP2, phase III transport processes may be coordinately regulated; however, the precise mechanisms are not clear.

Storage The distribution of organic xenobiotics to sites of storage are in most cases not directed, but are the results of simple chemical partitioning. Storage sites may be the target sites (e.g., paraquat in lung), or sites of noninteraction. Therefore, storage can only be considered a protective mechanism in the latter case. Major storage sites can include the following: plasma proteins (e.g., Cu is stored by ceruloplasmin [55]), liver and kidney (e.g., organic acids bind to Y protein/ligandin in liver), fat (e.g., chlordane, DDT, PCBs, dioxins), and bone (e.g., Pb). Xenobiotic sequestration inside the cell appears to be an uncommon protective mechanism. Doxorubicin (chemotherapy drug) was shown to be sequestered into small cellular vesicles whose concentration was correlated to the cellular resistance level to the drug [56]. This storage mechanism could participate in exocytosis and elimination. Glutathione S-transferase and other transferases contain lipophilic domains that can serve as binding sites for a number of exogenous compounds that are not substrates for reactions [57]. Organisms have few mechanisms for protection against metal contaminants. Metallothioneins (MTs) are ubiquitous low molecular weight proteins rich in cysteines with a high affinity for specific metals. They function in metal homeostasis by binding essential metals (e.g., Zn) and in defense by sequestering nonessential metals (e.g., cadmium). MTs have been identified in most tissues, and can be greatly induced in tissues that are active in metal uptake, storage, and excretion [58].

Toxicodynamically Derived Resistance Mechanisms

Toxicodynamically derived resistance refers to alterations in xenobiotic-receptor interactions. This can be manifest by alterations in target site, increased or

decreased concentrations of target molecules, or circumvention of target function. Whether any of these resistance mechanisms arises and is retained in a population depends on a number of factors, most importantly its presence in the population and the physiological cost of the mechanism to the organism. Variable resistance mechanisms will come into play on different time scales; for example, a single major resistance mechanism should arise first with some associated cost, a cost that can potentially be corrected by epistasis with subsequent less costly mechanisms.

Cellular Detoxification Many xenobiotics have similar modes of action, and the evolutionary chemical history of organisms has equipped them with various mechanisms in dealing with toxic effects they are likely to encounter. Many xenobiotics or their metabolites are electrophilic and highly reactive. For example, alkylating agents and drugs have lethal activity that is linked to electrophilic (quinines, cations) or radical species. The structure of GSH and its chemical characteristics have been described previously. The GSH thiol group performs a nucleophilic attack on the target electrophile and forms a stable conjugate that is nonreactive.

Target Site Alterations

Structural Modification In contrast to the above example, many xenobiotics (particularly therapeutic drugs) are targeted to a specific molecule or cellular component. An organism may resist the action of a xenobiotic due to target site modification that affords it an efficient resistance mechanism. Non-silent point mutations within structural genes are the most common cause of target-site resistance [59]. These changes can be brought about by alterations in amino acid sequences, the distances between critical amino acid residues, and protein topography. These mechanisms can be rapidly acquired when the xenobiotic has a unique cellular receptor or when the xenobiotic resembles the target's endogenous substrate. Structural mutations (non-silent) that give rise to this type of resistance to the effects of a given xenobiotic can incur costs to the organism; altered binding/catalysis by structural changes can lead to biological effects from reduced functionality of the target in its normal physiological roles. For selection of the mutations to occur, the resultant amino acid change

must reduce the binding of the xenobiotic without causing a significant loss of the primary function of the target site. The number of possible amino acid substitutions, therefore, can be very limited. It is not surprising that identical resistance-associated mutations are commonly found across highly diverged taxa [59]. The degree to which function is impaired by the resistance mutation is reflected in the fitness of resistant individuals in the absence of xenobiotic selection and the persistence of resistance in the field.

There are several classic examples of structural target site modification as a mechanism in insecticide resistance; resistance to organophosphates (OPs) and carbamates, legacy organochlorine insecticides, and the naturally derived insecticides (pyrethrins) are discussed as targets to components of the insect nervous system.

Acetylcholinesterase-based resistance has been well documented in numerous strains of mites, ticks, houseflies, and mosquitoes. The OPs and carbamates target acetylcholinesterase (AChE), an enzyme localized in the synapse that hydrolyzes the neurotransmitter acetylcholine once it has excited postsynaptic nerve membranes. The OP insecticides are converted to their oxon analogues via the action of monooxygenases before acting as AChE inhibitors. Alterations in AChE in OP- and carbamate-resistant insects result in a decreased sensitivity to inhibition of the enzyme by these insecticides [60]. The determination of kinetic constants of inhibition of AChE indicated that the binding affinities between AChE and the oxon metabolites of OPs or carbamates were lower in more resistant insect strains.

GABA receptors belong to a superfamily of neurotransmitter receptors that include nicotinic acetylcholine receptors. These receptors are formed by the oligomerization of five subunits around a central transmitter-gated ion channel. The GABA receptor in insects is a heteromultimeric gated chloride-ion channel, a widespread inhibitory neurotransmission channel in the insect's central nervous system and in neuromuscular junctions. The GABA_A receptor-chloride channel complex is known to be the target of cyclodiene insecticides. Dieldrin first enhances and then suppresses γ -aminobutyric acid (GABA)-induced chloride currents. An alanine-to-serine substitution in the putative channel-lining domain of the GABA receptor confers resistance to cyclodienes such as dieldrin in a broad range of

dieldrin-resistant insects [61]. The only variation in resistant insects is that glycine rather than serine can sometimes be the substituted amino acid residue. Despite the widespread switch away from the use of cyclodiene insecticides for agricultural and public health use the resistance allele is still found at relatively high frequencies in insect field populations. The insect GABA receptor is implicated as a site of action for some pyrethroids and avermectins as well as cyclodienes.

DDT and pyrethroids cause persistent activation of Na⁺ channels by delaying the normal voltage-dependent mechanism of inactivation. Insensitivity of Na⁺ channels to insecticide inhibition, also called knockdown or *kdr-like resistance*, was inferred from cross-resistance between DDT and pyrethroids, which act on the same site within the Na⁺ channel. Several mechanisms of resistance have been proposed and involve a reduction in the number of Na⁺ channels, changes in the fluidity of nerve membranes, or alterations in the binding characteristics between Na⁺ channels and insecticides. Changes associated with resistance are more variable than those seen in with GABA receptors but are still limited to a small number of regions on this large channel protein. The first mutation to be characterized in *kdr* insects was in the housefly. The Na⁺ channel of housefly contains 2,108 amino acids, which fold into 4 hydrophobic repeat domains. A leucine to phenylalanine point mutation in the S6 transmembrane segment of domain II produced 10- to 20-fold resistance to DDT and pyrethroids [62]. More than 500-fold resistance is found in "*super-kdr*" houseflies, which possess a mutation in a second methionine to threonine substitution further upstream in the same domain.

Overexpression of Target The up-regulation of target is also be a resistance mechanism by which the target is allowed to fulfill its biological function even in the presence of the xenobiotic at physiological concentrations and resultant toxicity. This is a compensatory mechanism for the portion of the target that is occupied. Thymidilate synthase (TS) is the major cellular target of 5-fluorouracyl (5-FU), a chemotherapy agent. 5-FU is activated to 5'-monophosphate-5-fluorouridine, which interrupts the action of TS, blocking the synthesis of the pyrimidine thymidine, a nucleotide required for DNA

replication, resulting in a thymidine deficiency and cell death in replicating cells. Resistant tumor cells show increased TS activity through increases in the amount of target per cell [63].

Down-regulation (Including Gene Disruption or Silencing) Finally, decreasing target site sensitivities can be achieved by down-regulation of receptor levels or responsiveness of signal transduction pathways. This can be problematic, as down-regulation of an important physiological process can lead to reduced function. Down-regulation can also occur for repressors; for example, AhR repressor mutations in regulatory factors causing disruption in silencer elements can result in overexpression of AhR and may carry a high cost due to dominant expression in the wrong tissues; like structural mutations, these types of mutations may arise early in resistance, but disappear when less deleterious alternatives come about. One example of down-regulation occurs in resistance to cancer chemotherapy. Topoisomerase II is a homodimeric enzyme that is essential for DNA replication since it modifies DNA topology by transient breakage of DNA and religation. During this process, an intermediate complex between the enzyme and DNA forms and is susceptible to several cytotoxic chemotherapy drugs (e.g., mitoxanthrone) that stabilize the complex leading to DNA fragmentation and chromosomal aberrations and rearrangements. In resistant tumor cells, topoisomerase II is down-regulated to levels just required for cell division, thereby reducing the amount of DNA damage caused by these drugs [63, 64].

Circumvention of Target This is a rare phenomenon of resistance but has been documented very early in organisms that have alternative mechanisms to bypass the affected site of action. An example of circumvention occurs in citrus-scale insects resistant to hydrogen cyanide. Widespread application of hydrocyanic acid (HCN formulation) was initially limited to the fumigation of valuable tree crops such as citrus fruits, which led to the development of other HCN-based insect and rodent control products for the fumigation of ships, stores, factories, and even residential buildings. The target molecule is cytochrome oxidase, in the fourth complex of the electron transport chain. HCN binds to Fe within this protein, preventing it from transferring electrons to oxygen, resulting in an inability to aerobically produce ATP.

Citrus-scale mites utilize an HCN-insensitive flavoprotein as an alternate electron carrier in the place of cytochrome oxidase, enabling electron transport [65].

Conclusions and Future Directions

Understanding the role of protection (tolerance and resistance) has numerous significant implications for the quality of life and the environment. It impacts the area of human health in disease treatment (e.g., chemotherapy), pharmacology (e.g., new drugs, individual susceptibilities, safety), and public health protection programs (disease and vector control). It is also crucial for many areas of the environment in determining sensitive and sentinel species, biomarker development, establishment of water quality guidelines, new chemical development, and risk assessment. As well, it has considerable economic ramifications (e.g., modern agriculture and pest resistance, cross-resistance, genetically modified food, biological control, integrated pest management programs). Few real tools for delaying or preventing the onset of resistance to chemicals such as pesticides and chemotherapeutic agents have yet to be put into practical use. Furthering basic scientific understanding of biological relationships (e.g., herbivory and allelochemicals), biological toxins and antidotes, development theories for resistance evolution, etc. are also of value.

Imagining future directions for a subject area as broad and multidisciplinary is fraught with potential omissions. Directions for more research are as diverse as the applications that would be supported. One thing is clear, however: With the enormous advances being made in molecular biology (e.g., toxicogenomics, bioinformatics, metabolomics) and its sophisticated tools, insights into the mechanisms and manipulations of protection against xenobiotics are fast approaching. These new tools and information will be transforming, and if researchers make a conscious effort to link molecular data to population or ecological levels, gains in this sphere of study will be vast.

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